

## Bacterial Group II Introns in a Deep-Sea Hydrothermal Vent Environment†

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**Group II introns are catalytic RNAs and mobile retrotransposable elements known to be present in the genomes of some nonmarine bacteria and eukaryotic organelles. Here we report the discovery of group II introns in a bacterial mat sample collected from a deep-sea hydrothermal vent near 9°N on the East Pacific Rise. One of the introns was shown to self-splice in vitro. This is the first example of marine bacterial introns from molecular population structure studies of microorganisms that live in the proximity of hydrothermal vents. These types of mobile genetic elements may prove useful in improving our understanding of bacterial genome evolution and may serve as valuable markers in comparative studies of bacterial communities.**

Deep-sea hydrothermal vents are some of the most productive ecosystems on our planet, even though they depend on microbial chemosynthesis instead of solar energy and photosynthesis (12, 16). Hot springs emanating from fractures in the earth's mantle at mid-oceanic ridges provide a rich source of minerals, which support a large microbial biomass able to fix carbon dioxide based on oxidizing or reducing sulfur compounds. These bacteria either serve as direct food for grazing or filter-feeding animals or are involved in complex and specific symbiotic relationships with invertebrates (6). The vent inhabitants have adapted to some of the most hostile life conditions on earth: steep temperature gradients (2 to >100°C), high concentrations of sulfides and heavy metals, and pressure in excess of 200 atm. Hydrothermal vents are not only biogeographic islands, separated in some cases by tens to hundreds of kilometers, but they are also highly ephemeral (years to decades) because of geological changes that terminate the supply of hydrothermal fluids. These factors pose unique challenges for their inhabitants in terms of colonization, dispersal, gene flow, and evolution (41).

Bacteria can occupy a variety of niches at hydrothermal vent sites, from the hot vent fluids and the hydrothermal plumes to the surfaces of rocks and animals in areas with diffuse vent flow that can be nearly as cold as the ambient deep-ocean temperature (2°C). Certain vent invertebrates (vestimentiferan tube worms, mollusks) form obligate endosymbiotic relationships with specific *γ-Proteobacteria* (6, 11). Other invertebrates (e.g., the shrimp *Rimicaris exoculata* and the polychaete *Alvinella pompejana*) are covered with epibiont bacteria that belong to the *ε-Proteobacteria* group (18, 33).

A number of approaches have been used to characterize the free-living microbial communities within deep-sea hydrother-

mal vent environments (21). These include cultivation enrichment in the laboratory (4, 35, 37), in situ colonization of growth chambers (7, 35), fluorescence in situ hybridization with rRNA probes (19), and sequencing of 16S rRNA genes (24, 27, 33). A major finding of those studies was the high abundance of *ε-Proteobacteria* among the free-swimming bacteria, in the mats that cover rocks and sediments, and as epibionts of metazoan animals that inhabit that environment. While the use of rRNA sequences has vastly improved our understanding of the microbial diversity at hydrothermal vents, there are some limitations to their use in population studies. The most problematic are biases and recombination events during PCR amplification, which can skew the biodiversity index of the sample or generate chimeric gene products (40). It has been shown that, in some organisms, the rRNA sequence can vary among genes located in different cistrons (36). RNA genes occur in all living organisms, but enormous species diversity in many microbial communities sometimes makes it difficult to identify and track minority members in molecular population structure investigations. As for studying intraspecific variations, as well as potential horizontal gene transfer events within microbial populations, new genetic markers could prove very useful.

Group II introns are a class of retrotransposable genetic elements that have been shown to engage in mobility events within bacterial populations (28). Their presence has been suggested to correlate with specific strains (17), but sequence data suggest that they can also spread horizontally across species (9). Group II introns are derived from ancient genetic elements that may have played a role in the early evolution of genomes. DNA corresponding to group II introns codes for a class of catalytic RNAs that interrupt bacterial genes, as well as genes from the organelles of fungi and photosynthetic eukaryotes (3, 26). Their splicing mechanism resembles that employed by the spliceosomal machinery during splicing of eukaryotic nuclear pre-mRNAs (26, 30). A widely accepted hypothesis is that group II and spliceosomal introns have an ancestor in common (5, 31). If this is true, group II introns must have served important roles in the evolution and shaping

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of early genomes, with possible direct connections to the "RNA world."

We are interested in understanding the biology of group II introns, their evolution, and their distribution among genomes. No such introns have been described in marine bacteria. The unique characteristics of hydrothermal vent bacteria and of that entire ecosystem made it a primary target in the search for possibly unusual group II introns. If group II introns exist in this environment, they could serve as a useful tool in studies of population dynamics and genetic exchanges across different niches, as well as between vent sites. By using a PCR approach, we investigated the presence of such introns in a bacterial mat, as well as in bacterial endosymbionts from the tubeworm *Riftia pachyptila* and the vent clam *Calyplogena magnifica*.

**Biological material.** Samples were obtained from three different microenvironments within a hydrothermal vent community (the East Wall site) located at 9°50'N, 104°17'W on the East Pacific Rise (EPR) at a depth of 2,500 m. The microenvironments included the surfaces of a basalt recruitment block and its attached fauna and the symbiont-containing tissues of the vestimentiferan tubeworm *R. pachyptila* and the vent clam *C. magnifica*. The basalt block with attached specimens of the hydrothermal vent mussel *Bathymodiolus thermophilus* was collected from a bed of mussels with the submersible *Alvin* (dive no. 3223). The ambient deep-water temperature in this region was ~2°C, and the temperature anomaly at the collection site was <5°C. The block was carried to the surface in closed containers with seawater and immediately processed. This minimized the risk of contamination with nonvent organisms. A bacterial mat sample consisting of filaments attached to the block and the mussel shells was collected with sterile utensils and frozen at -80°C. Gill tissue of *C. magnifica* and trophosome of *R. pachyptila* containing bacterial endosymbionts were dissected from animals collected at the same site and then frozen at -80°C.

**Bacterial diversity in the EPR vent sample.** Genomic DNA was extracted from the bacterial mat or animal tissue containing intracellular bacterial symbionts by following a standard protocol (1). To identify the major bacterial components of the mat, we amplified a 16S rRNA gene fragment with universal bacterial primers 8F (5'-AGAGTTTGATCMTGGC3') and 964R (5'-TGTGGTTTAATTCGA3') and the Expand Long Template DNA polymerase system (Roche). Amplicons were cloned with the PCR TOPO-XL kit (Invitrogen), and 12 clones were sequenced. The sequences were aligned with 36 other bacterial species, essentially covering the entire *Bacteria* domain, including representative sequences of other bacteria identified at hydrothermal vent sites. Phylogenetic analysis of the set of data was conducted with distances calculated in accordance with a GTR +  $P_{\text{invar}}$  +  $\Gamma$  maximum-likelihood model under the minimum-evolution criterion. Out of 12 bacterial rRNA phylotypes, 11 are related to rRNAs from  $\epsilon$ -*Proteobacteria* and one sequence represents a  $\delta$ -*proteobacterium*. A number of studies have shown that the majority of bacteria that form free-living communities at hydrothermal vents belong to a specific clade of  $\epsilon$ -*Proteobacteria* that appear to be endemic to these sites (4, 24, 33, 34). The abundance of  $\epsilon$ -*Proteobacteria* may reflect metabolic abilities that allow them to thrive in environments rich in sulfur compounds and heavy metals (4). Our data extend this taxonomic distribution previ-

ously reported for other vent sites, also vents near 9°N on the EPR. Similar to what has been observed in samples collected from other vent sites, the  $\epsilon$ -*proteobacterial* sequences cluster in subgroups, with identities of 90 to 98% between individual sequences. Details of the phylogenetic analysis and the phylogenetic trees are available upon request.

**PCR and cloning of bacterial group II introns.** To search for group II introns in the vent bacteria, we employed a PCR strategy that depends on several highly conserved sequences present in some group II introns. The substructure known as domain 5 is the most highly conserved domain in all group II introns. Some, but not all, group II introns contain an open reading frame (ORF) with strong similarity to reverse transcriptases (RTs). Introns that have a reading frame are readily identified by a PCR strategy using degenerate primers complementary to those conserved sequences. When both sequences are present in an intron, a 600- to 900-bp-long PCR product is obtained, depending on whether or not the intron reading frame contains a C-terminal endonuclease domain. This approach has successfully identified typical ORF-containing group II introns in various bacterial DNA samples (14, 15).

Group II intron fragments were amplified by using the approach and universal degenerate primers described by Ferat and Michel (15). These primers (RID1, 5'-TCCCTCCGAACCGTACGTGMNASTYTC3'; RID2, 5'-ACCGTATACGTAMGNTAYGCNGAYGA3') will amplify a fragment of any typical ORF-containing group II intron (not only bacterial). The PCRs contained 50 ng of bacterial DNA as the template and involved 30 amplification cycles (15 s at 94°C, 1 min at 50°C, and 2 min at 72°C) with *Taq* DNA polymerase. A single PCR product (~0.6 kb) was obtained when bacterial mat DNA was used as the template, and no product was detected in negative control reactions. We also did not obtain any amplification products from the *C. magnifica* and *R. pachyptila* symbiont DNAs.

The mat DNA PCR products were cloned into a pGEM-T vector (Promega), and eight individual clones were sequenced. All of the sequences contained a fragment of a group II intron, on the basis of the deduced encoded protein, and three distinct introns (EPR Vent-I1, -I2, and -I3) were identified. Out of the eight clones, four encoded intron I1, three encoded intron I2, and one encoded intron I3. There was no sequence variation between the clones encoding each intron. As predicted by our PCR strategy, the amplified intron fragments contain the C-terminal domain of the intron-encoded RT domain, as well as the maturase domain (X domain). Alignment of the amino acid sequences revealed 40 to 50% identity among the three proteins (Fig. 1). The closest relatives are the group II intron maturases encoded by the *Sinorhizobium meliloti* RmInt1 and *Escherichia coli* EcB introns (19 and 12% identity, respectively).

An inverse PCR approach was used to amplify full-length coding regions for the most abundant group II intron (EPR Vent-I1) of the vent bacterial DNA (15). One-microgram aliquots of bacterial mat DNA were digested with different restriction enzymes (*Bam*HI, *Eco*RI, and *Hind*III) and ligated in diluted solutions, favoring circularization. By using *Hind*III-digested and ligated DNA and PCR primers designed on the basis of the EPR Vent-I1 sequence, the entire intron 1 (2,017 nucleotides) was amplified, including short flanking sequences.

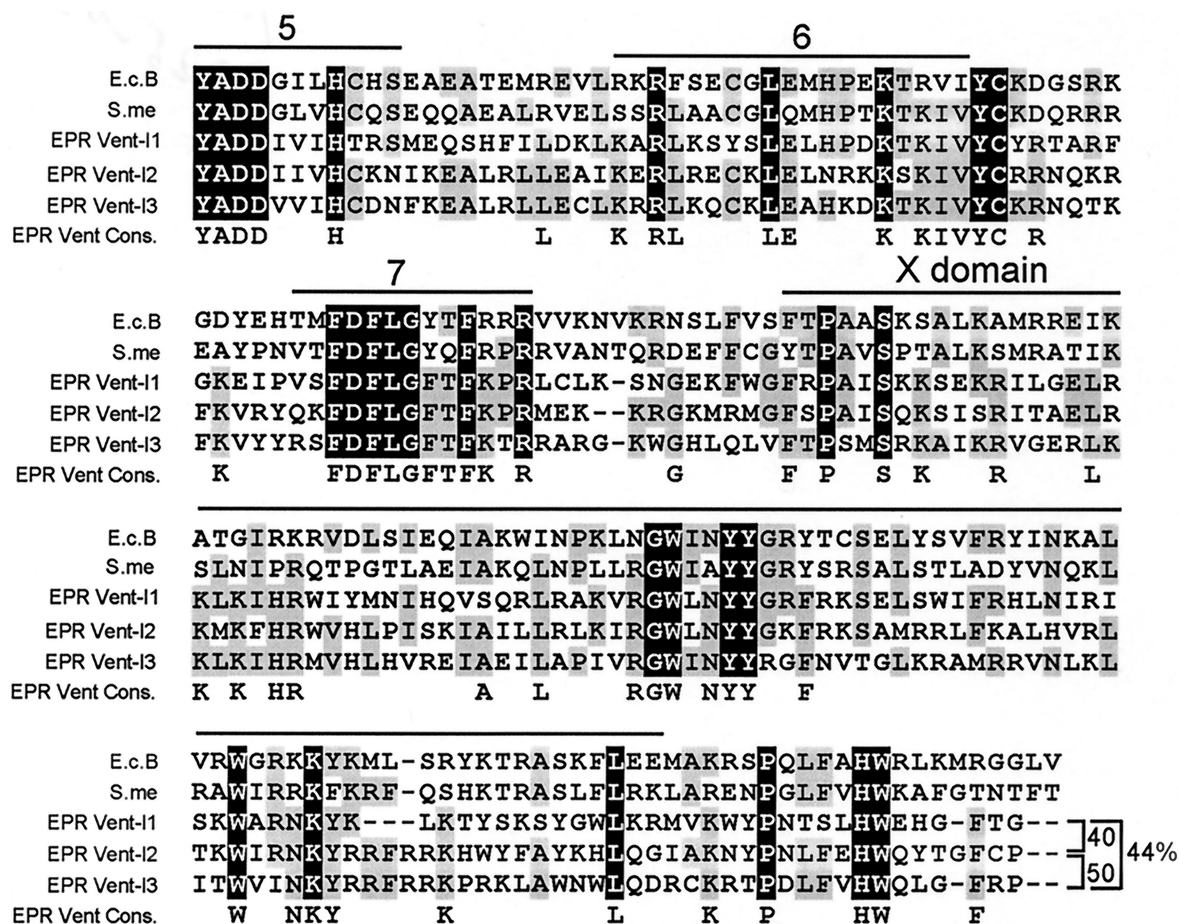


FIG. 1. Alignment of RT domains 5 to 7 and the X domains of the three EPR vent introns with the sequences of two bacterial group II introns, E.c.B (*E. coli* IntB) and S.me (*S. meliloti* RmInt1). Invariant residues are indicated in black blocks, and those that are at least 60% conserved are shaded. Also shown are the consensus (Cons.) sequence for the three EPR Vent introns and the percentages of identity between them.

As a final step, primers specific to those flanking sequences were used to amplify the entire EPR Vent-I1 sequence from the bacterial mat DNA with the Expand High Fidelity enzyme (Roche). The product was subcloned into a pCR-XL TOPO vector (Invitrogen), generating pCR-Vent-I1, and confirmed by sequencing.

**Analysis of EPR Vent-I1 ORF sequence.** The 386-amino-acid-long Vent-I1-encoded protein contains the RT and maturase domains and has no unusual sequence relative to other bacterial maturases. Similar to a number of other bacterial introns, there is no C-terminal endonuclease (Zn) domain. To better understand the relationship between the EPR Vent-I1 maturase protein and other group II intron maturases, we performed a phylogenetic analysis. The amino acid sequence of the EPR Vent-I1 RT/maturase was aligned with those of 25 other group II introns (18 bacterial, 6 algal, and 1 fungal) with ClustalW (38), and the alignment was manually refined. The sites that could not be reliably aligned were excluded. The resulting alignment contained 247 amino acids, covering the RT and X domains. A Bayesian phylogenetic analysis of this set of data was performed with the MRBAYES software (20). Four simultaneous MCMC chains run for  $10^6$  generations after the convergence of the likelihood values generated  $10^4$  trees. A

50% majority rule consensus tree was generated, and the bipartition values (percentage representation of a particular clade in the  $10^4$  trees) were recorded at the nodes. The set of data was also analyzed with the PHYLIP v3.6 package (13) under distance/neighbor joining and parsimony. Bootstrapping ( $10^3$  replicates) was performed with Seqboot. All methods yielded trees with virtually identical topologies. The Bayesian consensus tree (Fig. 2) shows the characteristic relationship between bacterial maturases and the eukaryotic mitochondrial and chloroplastic maturases reported by others (25, 42). The EPR Vent-I1 maturase is most closely related to the *S. meliloti* RmInt1 and *E. coli* IntB maturases. The statistical support for this relationship is robust under all of the phylogenetic methods used.

**Analysis of EPR Vent-I1 intron RNA sequence.** A secondary-structure model of the intron RNA was derived that was based on the overall conserved six-domain organization of group II introns (Fig. 3). The EPR Vent-I1 intron is A/U rich (60% A+U) and belongs to the class D bacterial group II introns (39). All of the characteristic substructures associated with these introns, as well as the pseudoknot tertiary contacts and the splice junctions, have been identified. The sequence of domain 5, a catalytically critical substructure of group II in-



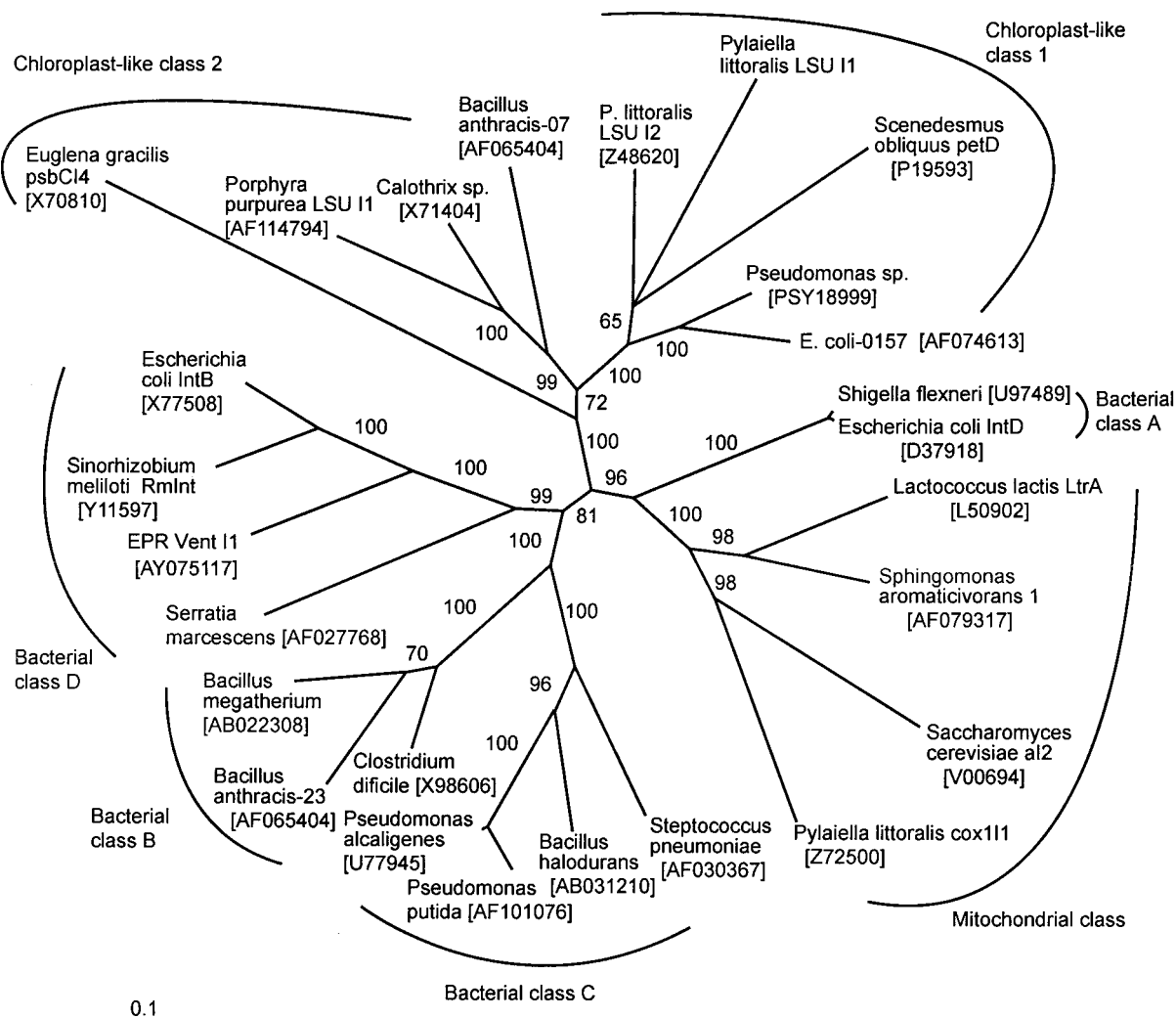


FIG. 2. Unrooted Bayesian consensus tree showing the relationship of the EPR Vent-I1 ORF with other ORFs (RT and X domains) from known bacterial introns and representatives of eukaryotic group II introns. The values represent the bipartition numbers (percent probability based on  $10^4$  trees). GenBank accession numbers are in brackets.

trons, is quite typical. One peculiarity of the structure of EPR Vent-I1 is the very long D1A. As with all bacterial group II introns, the maturase ORF of EPR Vent-I1 is fully contained within domain 4 and a ribosomal binding site was identified.

To determine whether the EPR Vent-I1 intron interrupts a protein gene with known homologues in the molecular databases, we performed BLAST searches with the 137-nucleotide spliced exon sequence. With this short sequence, we did not identify statistically significant matches. It is possible that the intron interrupts an intergenic region. Approximately half of the 30 or more known full-length bacterial group II introns do reside outside of ORFs (9, 25). The high frequency of group II intron insertions outside of coding regions in bacterial genomes, as well as the presence of numerous intron fragments, suggests that in bacteria, these introns can behave primarily as retroelements, being rapidly gained or lost (9).

**In vitro self-splicing and RT-PCR.** Because some bacterial and fungal group II introns are able to self-splice in vitro, we investigated the self-splicing potential of EPR Vent-I1. An

EPR Vent-I1 pre-mRNA was made by in vitro transcription with T7 RNA polymerase of a *Hind*III-linearized pCR-Vent-I1 plasmid template in a reaction containing [ $\alpha$ - $^{32}$ P]UTP (32). The labeled transcript was purified by electrophoresis in a denaturing polyacrylamide gel. Self-splicing reactions were done at 42°C in accordance with standard protocols (32) in low-salt buffer (50 mM HEPES [pH 7.3], 100 mM MgCl<sub>2</sub>) or high-salt (low-salt buffer supplemented with 1 M ammonium sulfate or 1 M KCl). The products were separated on 4% denaturing polyacrylamide gels and then subjected to PhosphorImager analysis. Initial experiments revealed that the level of EPR Vent-I1 self-splicing is much lower than that of a control intron, yeast mitochondrial intron aI5 $\gamma$ . Following 2-h-long incubations of labeled EPR Vent-I1 precursor RNA, no excised intron product was evident (data not shown). To test whether any splicing occurs, we performed RT-PCR experiments that can detect the other product of self-splicing, the ligated exons. Here, unlabeled transcript RNA was self-spliced for 30 min at 37 or 42°C in 40 mM HEPES (pH 7.3)–100 mM

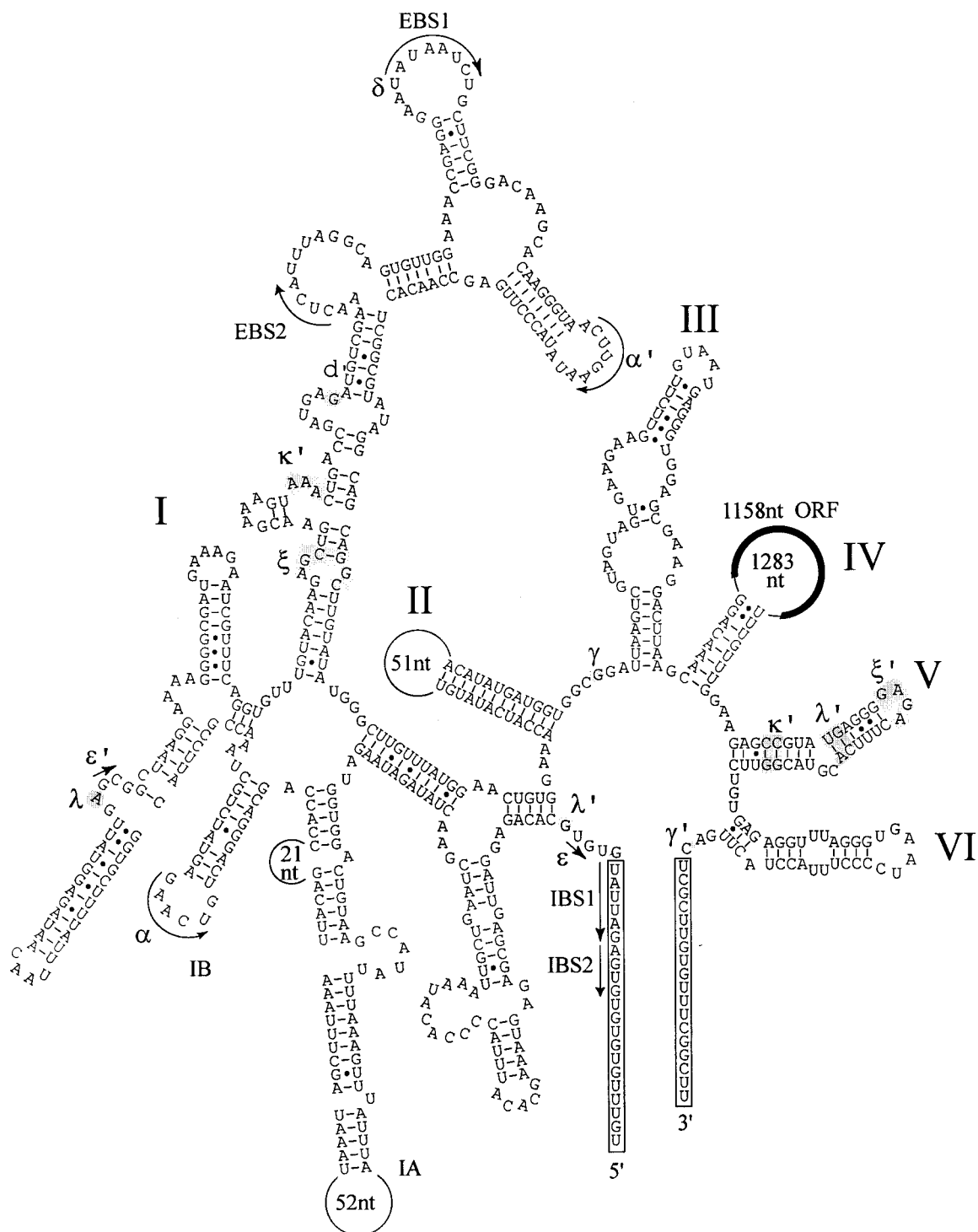


FIG. 3. Secondary-structure diagram of the EPR Vent-II intron RNA. The ORF in domain 4 is indicated by the thick line. Known tertiary interactions in group II introns are shaded or indicated by arrows. nt, nucleotides.

MgCl<sub>2</sub> (low-salt buffer) or in low-salt buffer supplemented with 1 M ammonium sulfate or 1 M KCl. The samples were then desalted by centrifugation in a Microcon 10 (Millipore) filter and precipitated with ethanol. RT-PCRs were done with exon-specific primers (VF, GCTGTGTCTTGAATGCT

CC; PCR-R, AGCTTGGTACCGAGCTCG GA) and the Titan RT-PCR kit (Roche) in accordance with the manufacturer's recommendations. The products were analyzed on 4% Metaphor agarose (FMC) gels. The product corresponding to the spliced exon was gel purified, cloned into a

pTrcHis2 TOPO vector (Invitrogen), and sequenced. The size and sequence of that product confirmed that splicing had occurred at the splice junctions predicted by the analysis of the intron structure (data not shown).

One difficulty with using new molecular markers to characterize microbial environmental samples is the need to associate a particular host organism with the molecular marker under investigation. The only way to accomplish that association is to physically determine the linkage of an environmental sequence for the new marker with a phylogenetically informative 16S rRNA. Although we are unable to establish which bacteria harbor each intron, it is possible that they belong within the highly predominant bacterial group  $\epsilon$ -*Proteobacteria*, which accounts for >90% of the sample phylotypes. They may, however, be encoded within the genomes of minor bacterial components of the mat or possibly within the genome of a single kind of bacterium. Limited amounts of sample DNA prevented us from identifying the host genome by hybridization or large-insert library construction.

Genome analysis has revealed a high level of horizontally transferred genes in bacteria and archaea from thermophilic microbial communities (22, 29). Within the dynamic hydrothermal vent environment, the microbial communities that occupy individual niches are constantly exposed to and in equilibrium with those of neighboring ones. Mobile genetic elements such as group II introns might take advantage of this environment to spread across taxa and between niches. The mobility of group II introns relies on their remarkable ability to undergo RNA-catalyzed reverse splicing into double-stranded DNA. This process has been characterized in detail for several yeast mitochondrial and bacterial group II introns (reviewed in references 2 and 23). Group II introns can also move to ectopic DNA sites (retrotransposition), a process that may have shaped genome evolution (8, 10). Such behavior has been documented for the RmInt1 intron in natural populations of *S. meliloti* (28). Recent analyses suggest that, in general, the intron RNAs have coevolved with the encoded maturases and that group II introns not only are inherited by vertical transmission but also move horizontally between genomes (9, 39, 42). The still relatively small number of known bacterial group II introns, as well as their propensity for horizontal transfer, complicates attempts to understand their direction of evolution. Analysis of bacterial communities that harbor numerous related group II introns, such as the one that we studied here, may provide important clues to better understand the biology and the evolutionary history of these mobile elements.

Group II introns could also serve as molecular tracers in bacterial communities, even without the knowledge of which bacteria serve as hosts. It has been previously recognized that 16S rRNA analyses do not provide a complete picture of the genetic and metabolic diversity of microbial assemblages. We are evaluating strategies for the use of these genetic elements to study the dynamics of populations at specific locations, as well to get a glimpse at potential exchanges between niches.

**Nucleotide sequence accession numbers.** The sequences reported here have been submitted to the GenBank database and assigned accession numbers AY075117 to AY075119 and AY075120 to AY075131.

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